OBSERVATION BY ^{13}C NMR OF METABOLITES IN DIFFERENTIATING AMOEBA

Trehalose storage in encysted *Acanthamoeba castellanii*

Roxanne DESLAURIERS, Harold C. JARRELL, R. Andrew BYRD and Ian C. P. SMITH
Division of Biological Sciences, National Research Council of Canada, Ottawa, Ontario, K1A OR6, Canada

Received 9 June 1980

1. Introduction

Acanthamoeba castellanii [1] is a useful organism for the investigation of eukaryotic cell differentiation at the chemical level. Protozoa show many properties characteristic of cells of higher organisms such as phagocytosis, pinocytosis [2,3] and osmoregulation [4]. *Acanthamoeba,* in particular, can undergo a certain degree of differentiation in that it can exist as a trophozoite or a cyst depending on the growth medium [5–7].

By virtue of its non-destructive nature, high resolution NMR spectroscopy is ideally suited to the study of the chemical composition, and changes thereof, in living organisms. The selectivity and resolution of the method with respect to the chemical nature of cellular components renders it attractive for studies of cell physiology [8,9].

In the course of our studies of the osmoregulatory properties of *Acanthamoeba castellanii* it became evident that washed cells contain large amounts of a low molecular weight sugar, and that the nature of the sugar changes on going from the vegetative to the encysted state. We have identified the sugar in encysted *Acanthamoeba castellanii* as α,α-trehalose using ^{13}C NMR and verified the assignment by thin-layer chromatography. We believe this to be the first observation of α,α-trehalose as a metabolic product in *Acanthamoeba,* in both the vegetative and encysted organism.

2. Materials and methods

Amoeboid cells of *Acanthamoeba castellanii* (Neff strain) [1] were obtained from Dr J. E. Thompson, University of Waterloo, Canada. Growth conditions were as in [10]. Trophozoites were obtained from 7-day-old cultures. Encysted cells were obtained by adding 50 mM $MgCl_2$ to 7-day-old cultures [6,7], or by transfer to encystment medium maintaining constant pH during encystment [5]. Ampicillin (40 mg/l) was added to cultures which were to be maintained for long periods. Cells were harvested by centrifugation at 800 × *g* for 5 min and washed 3 times in water or 0.1 M KCl to remove traces of growth medium.

Extracts of cells were performed by the method of Bligh and Dyer [11]. The aqueous phase was placed on a rotary evaporator to remove methanol and then freeze-dried. The chloroform-containing phase was dried on a rotary evaporator, placed under vacuum for 12 h to remove traces of solvent and redispersed in chloroform for NMR spectroscopy. Whole cells for ^{13}C NMR were freeze-dried from a washed pellet and resuspended in aqueous medium.

^{13}C NMR spectra were obtained on Varian CFT-20 and Bruker CXP-300 spectrometers operating at 20 and 75 MHz, respectively. Samples were run in 10 mm and 15 mm sample tubes at 30°C, with proton-decoupling. All chemical shifts are reported with respect to external tetramethylsilane.

3. Results and discussion

Fig.1 shows the natural abundance ^{13}C NMR spectrum of cysts of *Acanthamoeba castellanii.* The cysts were from a 1 liter 16-day-old, culture to which 50 mM $MgCl_2$ was added at 7 days; 200 ml culture were centrifuged and resuspended in 2 ml D_2O for NMR. Identical spectra were obtained from

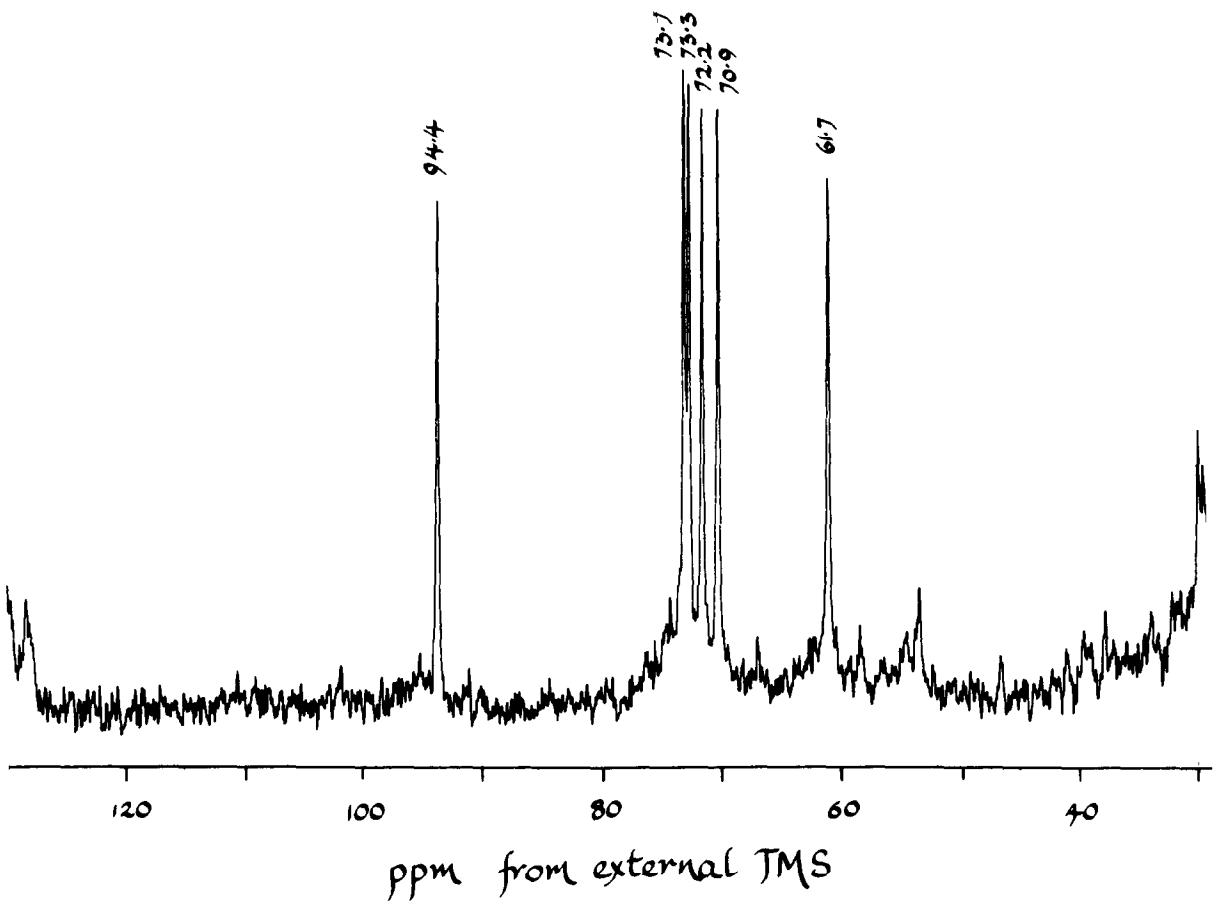

Fig.1. ^{13}C NMR spectrum of encysted *Acanthamoeba castellanii:* observed at 75 MHz, 10 kHz sweepwidth, 16 000 data points, acquisition time 0.8 s, 30 μs pulsewidth (90°), complete proton noise decoupling, 23 000 scans, 2 Hz line broadening, 3 point smoothing, 30°C, in water at pH 6.0, sample tube 15 mm diam.

cysts which had been transferred to encystment medium at 7 days and examined by NMR at 21 days. A number of sharp resonances characteristic of material which is freely rotating and of low M_r ($\lesssim 2000$) are observed in the central part of the spectrum, in addition to weaker resonances at lower and higher field. The number of sharp resonances, as well as their chemical shifts, suggest the possibility that they arise from monomeric or oligomeric hexose. The less intense signals which are superimposed on a very broad component may arise from less mobile cellular components such as protein or lipid aggregates.

To investigate whether vegetative cells showed similar simple spectra, we examined freeze-dried vegetative cells or Bligh-Dyer [11] extracts of vegetative cells to insure that over the time course of the experiment lysis of cells under anaerobic conditions would not lead to erroneous measurements. Fig.2 shows the components isolated from the aqueous phase of a Bligh-Dyer extract of 7-day-old *Acanthamoeba castellanii.* The spectra contain the same narrow resonances seen in the cysts, as well as additional narrow resonances. No broad resonances are seen at high or low field. Fig.3 shows the ^{13}C NMR spectrum of whole freeze-dried cells resuspended in aqueous medium. The resonances are less well defined than in the spectrum of the Bligh-Dyer extract, and in addition there is a clearly resolved resonance at 100.7 ppm which was not present in the spectra of either of the cysts or the cell extract.

In all the above spectra the pattern as well as the chemical shift distribution of the narrow resonances are characteristic of sugars [12–15]. Among the possible contributions to the ^{13}C NMR spectrum are glucose, cellulose and glycogen. The presence of glucose

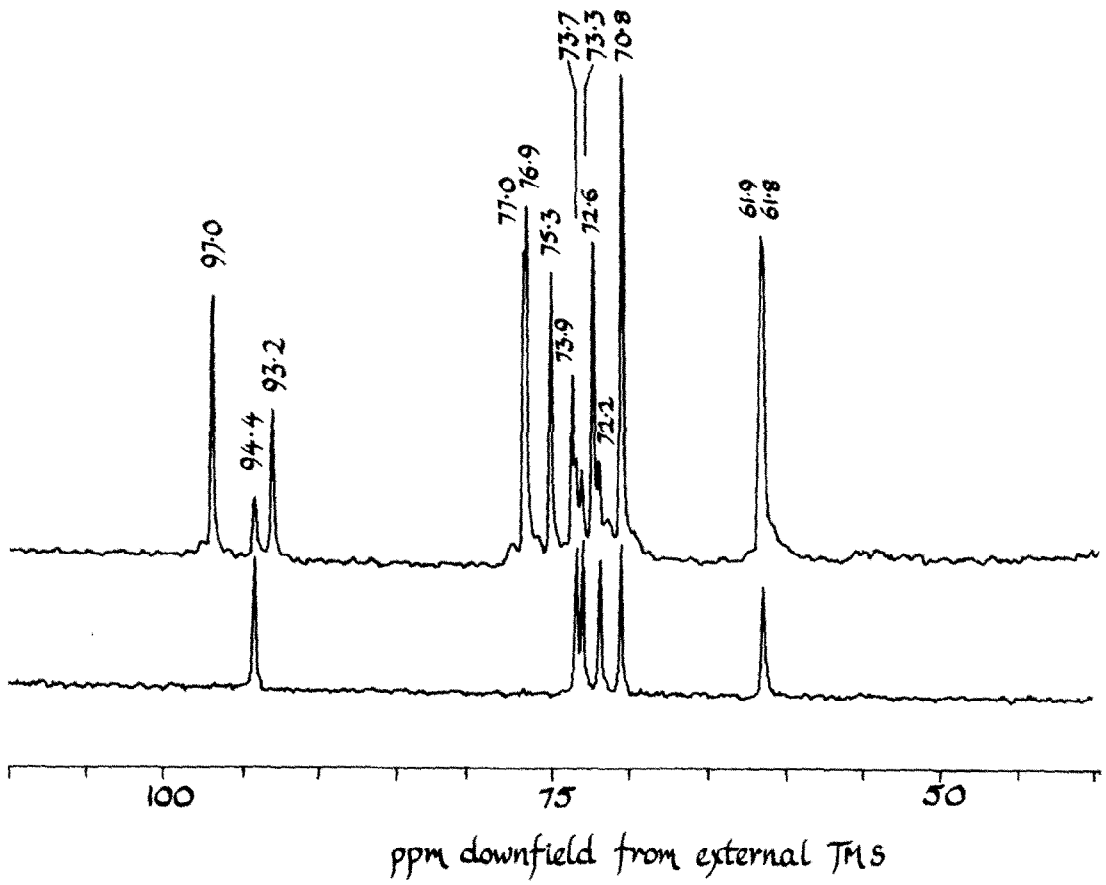

Fig.2. Upper spectrum: ^{13}C NMR spectrum of the aqueous phase from a Bligh-Dyer extraction of 7-day-old *Acanthamoeba castellanii:* observed at 20 MHz, 5 kHz sweepwidth, 5000 data points, acquisition time 0.5 s, 15 μs pulsewidth (60°), 800 μs α-delay (the time between the pulse and the start of data acquisition), complete proton noise decoupling, 8319 scans, 30°C, in D_2O at pH meter reading 6.4, sample tube 10 mm diam. Lower spectrum: ^{13}C NMR spectrum of α,α-trehalose in aqueous solution; spectral acquisition parameters are as above.

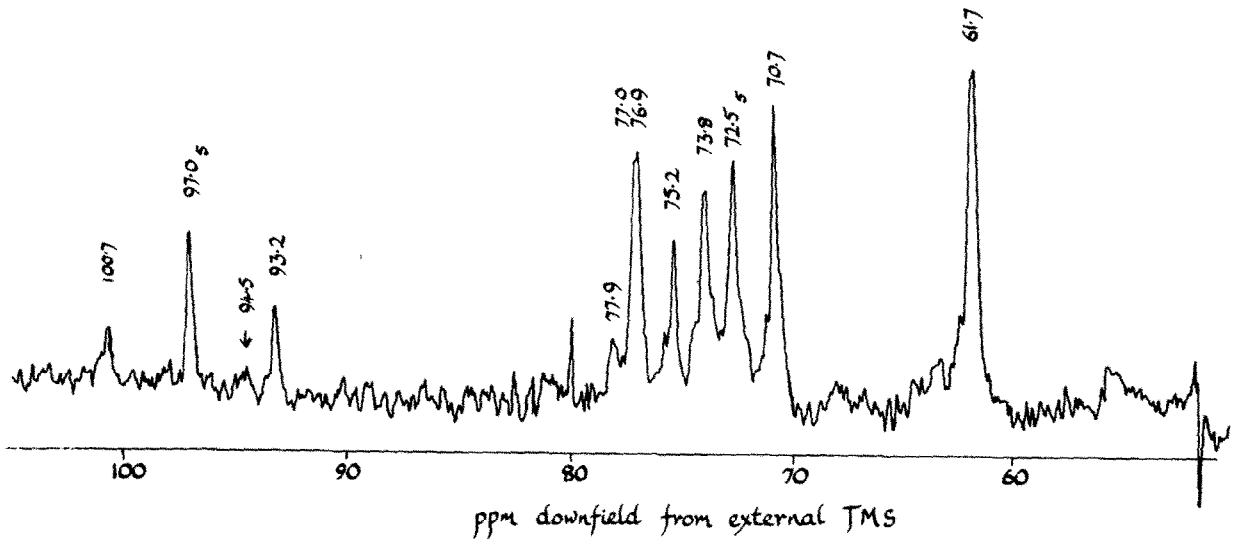

Fig.3. ^{13}C NMR spectrum of freeze-dried whole vegetative cells of *Acanthamoeba castellanii:* observed at 20 MHz, 4 kHz sweepwidth, 4000 data points, 8000 Fourier transform, acquisition time 0.5 s, 12 μs pulsewidth (45°), 800 μs α-delay, proton noise decoupling, 160 000 scans, 30°C in D_2O at a pH meter reading of 6.5, sample tube 10 mm diam.

Table 1
^{13}C Chemical shifts of glucose and glucose oligomers

Sugar	Anomer	Unit[a]	C-1	C-2	C-3	C-4	C-5	C-6	[Ref.]
Glucose	α		93.1	72.5	73.8	70.7	72.5	61.7	[12]
	β		97.0	75.2	77.0	70.7	76.8	61.8	
Trehalose	α,α		94.4	73.3	73.7	70.9	72.2	61.7	[c]
Maltose	α	*r*	93.1	72.5	74.4	78.3	71.2	62.0	[12]
	β	*r*	97.1	75.2	77.3	78.1	75.7	61.8	
		n	100.8	73.8	74.1	71.5	72.8	61.7	
Isomaltose	α	*r*	93.4	73.0	74.3	71.3	71.3	67.1	[12]
	β	*r*	97.3	75.3	77.2	70.8	75.5	67.1	
		n	99.3	73.3	74.3	70.8	72.7	61.8	
Maltotriose	α	*r*	93.4	73.4	74.7	78.5	71.6	62.4	[13]
	β	*r*	97.1	75.9	77.8	78.9	76.3	62.4	
		m	101.1	73.1	74.7	78.5	72.7	62.4	
		n	101.1	74.3	75.1	71.2	73.4	62.4	
Cellobiose	α	*r*	93.2	72.9	72.9	80.1	71.6	61.8	[13]
	β	*r*	97.1	75.7	76.1	80.1	75.7	61.8	
		n	103.9	74.7	77.2[b]	71.1	77.4[b]	62.4	

[a] *r*, reducing unit; *n*, non-reducing unit; *m*, middle unit
[b] Assignments could be reversed; [c] determined in this laboratory

Table 2
^{13}C Chemical shifts of sugars in *Acanthamoeba castellanii*[a]

Whole cysts aqueous solution		Trophozoite Bligh-Dyer extract aqueous phase[b]			Trophozoite freeze-dried cells aqueous suspension		
δ	Assignment	δ	Assignment		δ	Assignment	
94.4	T C-1	97.0	G(β)	C-1	100.7	M	
73.7	T C-3	94.4	T	C-1	97.1	G(β)	C-1
73.3	T C-2	93.2	G(α)	C-1	94.5[c]	T	C-1
72.2	T C-5	77.0	G(β)	C-3	93.2	G(α)	C-1
70.9	T C-4	76.9	G(β)	C-5	77.9	M	
61.7	T C-6	75.3	G(β)	C-2	77.0	G(β)	C-3
		73.9	G(α)	C-3	76.9	G(β)	C-5
		73.7	T	C-3	75.2	G(β)	C-2
		73.3	T	C-2	73.8	G(α)	C-3
		72.6	G(α)	C-2,C-5	72.5	G(α)	C-2,C-5
		72.2	T	C-5	70.7	G(α,β)	C-4
		70.8	G(α,β)	C-4	61.7	G(α,β)	C-6
			T	C-4			
		61.9	G(α,β)	C-6			
		61.8	T	C-6			

[a] T, trehalose (α,α); G, glucose; M, (1→4)-*O*-α-D glucopyranosyl linkage such as in maltose or higher oligomer thereof
[b] Trehalose:α-glucose:β-glucose; Ratio 2:5:8; [c] Weak intensity

in the washed cells might be expected as a result of uptake from the growth medium by pinocytosis and phagocytosis, the characteristic nutritive mechanisms [5] for *Acanthamoeba*. Cellulose has been reported as a major consituent of the cyst [16], while glycogen is found in the trophozoite and is believed to be degraded to glucose prior to formation of cellulose in the cyst [17].

Table 1 shows the ^{13}C chemical shifts of glucose and some naturally-occurring glucose oligomers, dissolved in aqueous solution [12,14]. Table 2 shows the major ^{13}C resonances from *Acanthamoeba castellanii* in the encysted and vegetative states. Comparison of the chemical shifts in tables 1 and 2 allows us to assign all the resonances in the encysted state of *Acanthamoeba* to α,α-trehalose (α-D-glucopyranosyl α-D-glucopyranoside). The aqueous phase of the Bligh-Dyer extract of the vegetative cells shows only glucose (α- and β-anomers) and α,α-trehalose, the latter representing 10–15% of the total. The spectrum of the freeze-dried whole cell shows well-resolved resonances; however, in addition to glucose and trace amounts of α,α-trehalose we observe resonances characteristic of maltose (4-*O*-α-D-glucopyranosyl D-glucopyranose) or a higher oligomer such as maltotriose [*O*-α-D-glucopyranosyl (1→4)-*O*-α-D-glucopyranosyl (1→4)-D-glucopyranose]. Maltose and maltotriose could be terminal residues of glycogen or products of glycogen metabolism. We do not find any evidence for the presence of cellobiose (4-*O*-β-D-glucopyranosyl D-glucopyranose) which has a characteristic resonance at 103.7 ppm. This implies that we are not observing any oligomeric precursors of cellulose. High M_r components with long rotational correlation times would not be observed under the present spectrometer operating conditions [18].

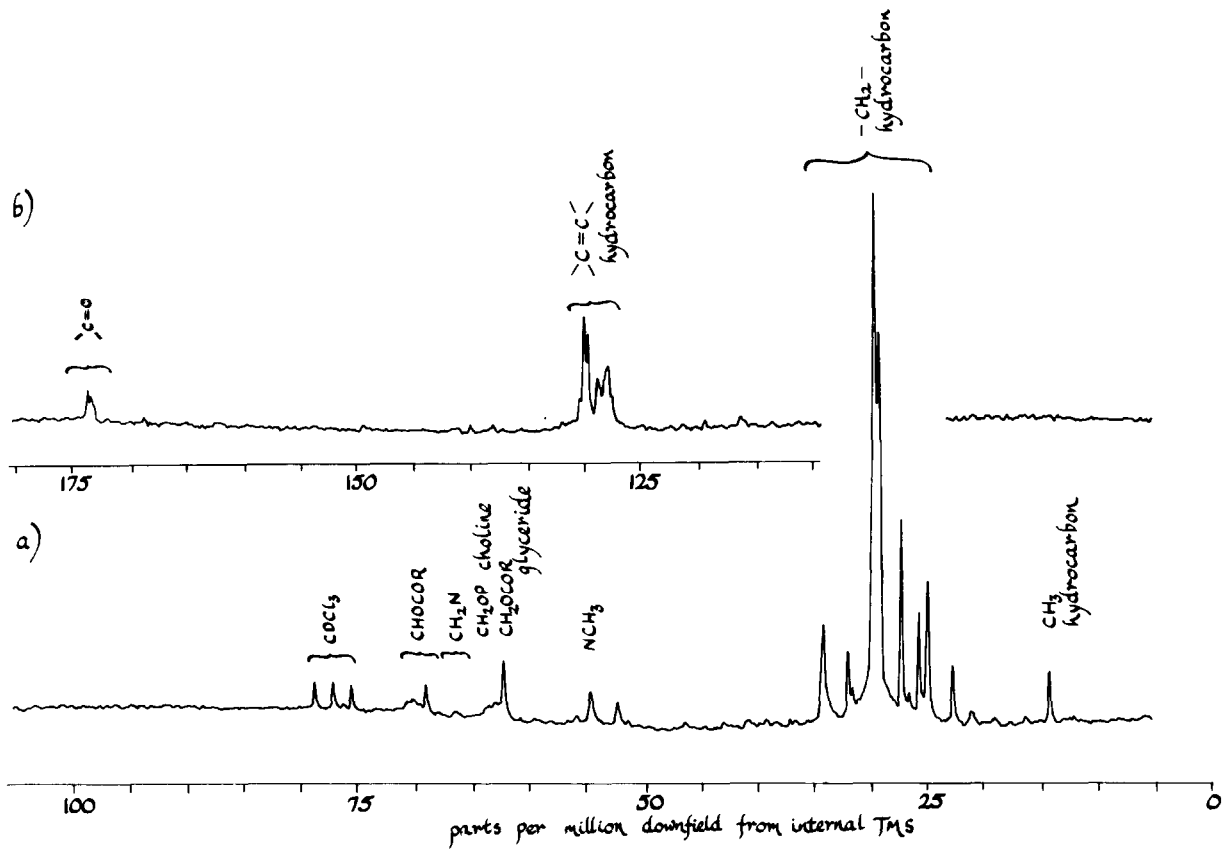

Fig.4. ^{13}C NMR spectrum of the organic phase from a Bligh-Dyer extract of vegetative cells of *Acanthamoeba castellanii*: observed at 20 MHz, 5 kHz sweep width, 5000 data points, 8000 Fourier transform, acquisition time 0.5 s, 15 μs pulsewidth (60°), 800 μs α-delay, complete proton noise decoupling, 7172 scans, 30°C, in $CdCl_3$, sample tube 10 mm diam. (a) High field portion of spectrum; (b) low field portion of spectrum.

α,α-Trehalose was further identified in cellular extracts by thin-layer chromatography on silica gel in the solvent system *n*-butanol/pyridine/water (10:3:3), showing the same mobility as an authentic sample of α,α-trehalose.

The weaker resonances in the whole cysts as well as in the freeze-dried cells can be identified; they arise mainly from the lipids. Fig.4 shows a ^{13}C NMR spectrum of the organic phase of a Bligh-Dyer extract of vegetative cells. All the resonances can be assigned [19] by assuming the mixture contains the major phospholipids found in *Acanthamoeba*, i.e., phosphatidylcholine (45%) and phosphatidylethanolamine (33%) [20], and that the main fatty acid chains are oleic acid (40–50%) and longer polyunsaturated fatty acids (20–30%) [20], as modeled by linoleic acid. Fatty acid analyses have been performed on trophozoites in this laboratory and found to be in general agreement with those reported in [20].

4. Conclusion

α,α-Trehalose has been reported as a storage sugar in a number of Protista and Metazoa. Among the higher organisms it has been extensively studied in *Nematoda* [21] and *Arthropoda* (Insecta [22,23]). In the Protista, fungi [24], slime molds [25] and protozoa [26] have been shown to store α,α-trehalose. To our knowledge the only report of α,α-trehalose in protozoa is in *Euglena* (class Mastigophora [26]). It is believed that trehalose can serve as a reserve of carbohydrate and energy, but the quantities available in *Euglena* are too small to account for the full energy reserve [26]. We have seen that in *Acanthamoeba castellanii* (class Sarcodina) trehalose appears to be the only oligomeric sugar present in the encysted form. This sugar can also be observed in the trophozoite; however glucose, and to a lesser extent maltose or oligomers thereof, are predominant. It would appear that trehalose remains fairly stable in encysted cells, as spectra obtained from 16-day-old encysted cells and 77-day-old cysts are similar, whereas the levels of nucleotide phosphates, as monitored by ^{31}P NMR, have changed considerably (R. D., H. J. J., R. A. B., I. C. P. S., in preparation).

This study demonstrates clearly the utility of NMR of ^{13}C in natural abundance to monitor metabolic products in living systems. The use of ^{13}C-labelled trehalose or glucose should permit the study of the metabolic fate of trehalose in cysts which are permitted to de-encyst and develop in normal nutritive media, delineating further the role of trehalose as a carbohydrate and energy reserve.

References

[1] Neff, R. J. (1957) J. Protozool. 4, 176–182.
[2] Chambers, J. A. and Thompson, J. G. (1976) J. Gen. Microbiol. 92, 246–250.
[3] Bowers, B. (1977) Exp. Cell Res. 110, 409–417.
[4] Drainville, G. and Gagnon, A. (1973) Comp. Biochem. Physiol. 45 A, 379–388.
[5] Neff, R. J., Ray, S. A., Benton, W. F. and Wilborn, M. (1964) in: Methods in Cell Physiology, (Prescott, D. M. ed) vol. 1, pp. 55–83, Academic Press, New York.
[6] Chagla, A. H. and Griffiths, A. J. (1974) J. Gen. Microbiol. 85, 139–145.
[7] Stratford, M. P. and Griffiths, A. J. (1978) J. Gen. Microbiol. 108, 33–37.
[8] Den Hollander, J. A., Brown, T. R., Ugurbil, K. and Shulman, R. G. (1979) Proc. Natl. Acad. Sci. USA 76, 6096–6100.
[9] Norton, R. S. (1979) Comp. Biochem. Physiol. 63B, 67–72.
[10] Thompson, J. E. (1977) in: Methods in Cell Biology (Prescott, D. M. ed) vol. 15, pp. 303–323, Academic Press, New York.
[11] Bligh, E. G. and Dyer, W. J. (1959) Can. J. Biochem. Physiol. 37, 911–917.
[12] Colson, P., Slessor, K. N., Jennings, H. J. and Smith, I. C. P. (1975) Can. J. Chem. 53, 1030–1037.
[13] Usui, T., Yamaoka, N., Matsuda, K., Tuzimura, K., Sugiyama, H. and Seto, S. (1973) J. Chem. Soc. Perkin I, 2425–2432.
[14] Wenkert, E. and Hagaman, E. W. (1976) J. Org. Chem. 14 70.
[15] Heyraud, A., Rinaudo, M., Vignon, M. and Vincendon, M. (1979) Biopolymers 18, 167–185.
[16] Neff, R. J., Benton, W. F. and Neff, R. H. (1964) J. Cell. Biol. 23, 66A.
[17] Weisman, R. A. (1976) Ann. Rev. Microbiol. 30, 189–219.
[18] Seiter, C. H. A., Feigenson, G. W., Chan, S. I. and Hsu, M. (1972) J. Amer. Chem. Soc. 94, 2535–2537.
[19] Barton, P. G. (1975) Chem. Phys. Lipids 14, 336–343.
[20] Ulsamer, A. G., Wright, P. L., Wetzel, M. G. and Korn, G. D. (1971) J. Cell. Biol. 51, 193–215.
[21] Wilson, A. G. (1976) Z. Parazitenk. 49, 243–252.
[22] Wyatt, G. R. and Kalf, G. F. (1957) J. Gen. Physiol. 40, 833–847.
[23] Friedman, S. (1978) Ann. Rev. Entomol. 23, 389–407.
[24] Blumenthal, H. J. (1976) in: The Filamentous Fungi, vol. 2, Biosynthesis and Metabolism (Smith, J. E. and Berry, D. R. eds) pp. 292–307, Halsted Press/Wiley, New York.
[25] Wilson, J. B. and Rutherford, C. L. (1978) J. Cell. Physiol. 94, 37–46.
[26] Marzullo, G. and Danforth, W. F. (1969) J. Gen. Microbiol. 55, 257–266.